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Neural cells in the esophagus respond to glial cell line-derived neurotrophic factor and neurturin, and are RET-dependent

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Abstract

Glial cell line-derived neurotrophic factor (GDNF) is expressed in the gastrointestinal tract of the developing mouse and appears to play an important role in the migration of enteric neuron precursors into and along the small and large intestines. Two other GDNF family members, neurturin and artemin, are also expressed in the developing gut although artemin is only expressed in the esophagus. We examined the effects of GDNF, neurturin, and artemin on neural crest cell migration and neurite outgrowth in explants of mouse esophagus, midgut, and hindgut. Both GDNF and neurturin induced neural crest cell migration and neurite outgrowth in all regions examined. In the esophagus, the effect of GDNF on migration and neurite outgrowth declined with age between E11.5 and E14.5, but neurturin still had a strong neurite outgrowth effect at E14.5. Artemin did not promote neural migration or neurite outgrowth in any region investigated. The effects of GDNF family ligands are mediated by the Ret tyrosine kinase. We examined the density of neurons in the esophagus of $Ret^{-/-}$ mice, which lack neurons in the small and large intestines. The density of esophageal neurons in $Ret^{-/-}$ mice was only about 4% of the density of esophageal neurons in $Ret^{+/-}$ and $Ret^{+/+}$ mice. These results show that GDNF and neurturin promote migration and neurite outgrowth of crest-derived cells in the esophagus as well as the intestine. Moreover, like intestinal neurons, the development of esophageal neurons is largely Ret-dependent.

Keywords: GDNF; Neurturin; Cell migration; Neurite outgrowth; $Ret^{-/-}$; Esophagus; Gastrointestinal tract

Introduction

The enteric nervous system is an extensive system of neurons and glial cells within the wall of the gastrointestinal tract. Most enteric neurons arise from neural crest cells that emigrate from the vagal level of the neural axis (post-otic hindbrain adjacent to somites 1–7) (Burns and Le Douarin, 1998; Le Douarin and Teillet, 1973; Yntema and Hammond, 1954).

The glial cell line-derived neurotrophic factor (GDNF) signaling pathway is essential for the development of the enteric nervous system in most regions of the gastrointestinal tract. GDNF acts at a receptor complex consisting of a ligand binding molecule, GFRα1, and a receptor tyrosine kinase, Ret (Airaksinen et al., 1999). Mice lacking GDNF, GFRα1, or Ret lack enteric neurons in the small and large intestines (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994; Tomac et al., 2000). GDNF appears to play multiple roles in enteric neuron development, including survival, proliferation, and differentiation (Chalazonitis et al., 1998; Focke et al., 2001, 2003; Gianino et al., 2003; Hearn et al., 1998; Shen et al., 2002; Taraviras et al., 1999; Worley et al., 2000). In addition, GDNF is chemoattractive to enteric neural crest-derived cells, and appears to play an important role in promoting the migration of vagal neural crest cells into and along the gastrointestinal tract (Barlow et al., 2003; Iwashita et al., 2003; Kruger et al., 2003; Natarajan et al., 2002; Young et al., 2001). Despite the absence of enteric neurons in the small and large

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intestines of mice with null mutations in *Gdnf*, *Gfrα1*, or *Ret*, enteric neurons are reported to be present in the esophagus of $Ret^{-/-}$ mice, and, therefore, it has been proposed that the development of neurons in the esophagus is Ret-independent (Durbec et al., 1996). Thus, enteric neuron precursors in different parts of the gastrointestinal tract may differ in their requirements for GDNF family members.

In addition to GDNF, two other members of the GDNF family of ligands are expressed in the developing gastrointestinal tract, neurturin and artemin. The effects of neurturin are mediated by a receptor complex consisting of its preferred co-receptor, GFRα2, and Ret, and the receptor complex for artemin consists of GFRα3 and Ret, although neurturin and artemin may also bind weakly to GFRα1 (Airaksinen and Saarma, 2002). Neurturin is expressed throughout the developing gastrointestinal tract (Golden et al., 1999; Widenfalk et al., 1997; Xian et al., 1999), whereas artemin is only expressed in the esophagus (Enomoto et al., 2001). Neurturin promotes the proliferation and differentiation of enteric neural crest-derived cells in vitro (Heuckeroth et al., 1998; Taraviras et al., 1999). Nonetheless, mice with null mutations in neurturin or GFRα2 have neurons present in normal numbers throughout the gastrointestinal tract, but show a decreased density of excitatory nerve fibres in the circular muscle (Gianino et al., 2003; Heuckeroth et al., 1999; Rossi et al., 1999, 2003). Studies of mice with a null mutation in artemin or GFRα3, the binding molecule for artemin, have not reported any defects in the enteric nervous system (Honma et al., 2002; Nishino et al., 1999). The effects of neurturin and artemin on enteric neural crest cell-derived migration have not previously been examined.

In this study, we examined the effect of GDNF, neurturin, and artemin on neural migration and neurite outgrowth from neural precursors in different regions of the E10.5–E14.5 gastrointestinal tract (esophagus, midgut, and hindgut). Our results show that both GDNF and neurturin induce neural migration and neurite outgrowth in the esophagus as well as the intestine, but the effect of GDNF declines with age. Although artemin is expressed in the esophagus, artemin did not induce migration or neurite outgrowth in the esophagus or any gastrointestinal region examined. In addition, although mice lacking Ret have been reported to possess enteric neurons in the esophagus, the density of enteric neurons in the esophagus of $Ret^{-/-}$ mice has not been previously been quantified. We show that the density of neurons in the esophagus of $Ret^{-/-}$ mice is dramatically (about 25 times) lower than that in $Ret^{+/-}$ or $Ret^{+/+}$ mice, suggesting that the development of enteric neurons in all regions of the gastrointestinal tract is largely Ret-dependent.

Materials and methods

Embryonic BALB/c mice were used for all organ culture experiments. The day at which a vaginal plug was found was designated E0.5. Pregnant mothers were killed by cervical dislocation, and the embryos removed using aseptic conditions.

Suspension (catenary) culture

Segments of esophagus, midgut, and hindgut from E11.5 mice, esophagus from E10.5, and midgut from E12.5 mice were dissected. The entire esophagus, which was 1.2–1.5 mm long, was dissected and explanted. Explants of midgut were from the caudal region, rostral to the caecum, and include regions that will develop into the ileum and jejunum. A "V" was cut into a 3 × 3-mm piece of Millipore filter paper using a scalpel, and an explant was suspended across the "V" as described previously (Hearn et al., 1999; Fig. 1). The orientation of the hindgut explant was indicated by removing the corner of the filter paper adjacent to the caudal end. The whole preparation, including the filter paper and the attached explant, was placed in a Terasaki well in 20 μl of tissue culture medium and cultured in a 5% CO_2 incubator at 37°C for 4 days. The tissue culture medium contained DMEM (Trace Scientific Ltd., Noble Park, Victoria, Australia) with 10% fetal bovine serum, 2 mM L-glutamine, 0.075% sodium bicarbonate, and penicillin/streptomycin sulfate solution. GDNF, artemin, or neurturin (all at 100 ng/ml, PeproTech Inc., Rocky Hill, NJ, USA) was added to the culture medium of some explants. The culture medium was changed after 2 days.

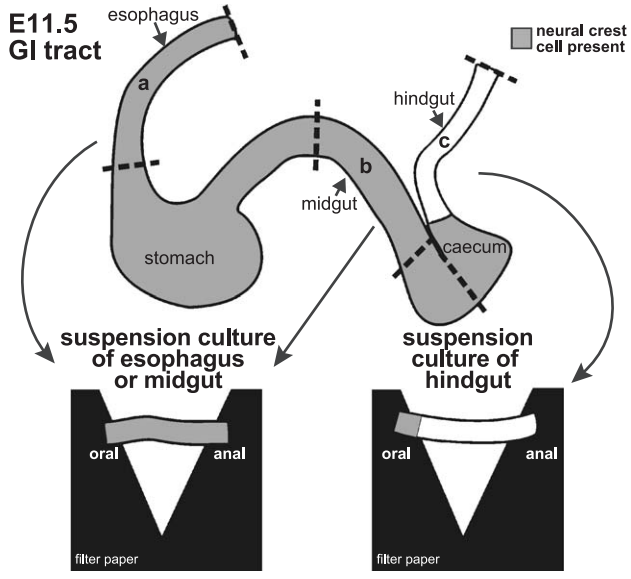

Fig. 1. Diagram of the gastrointestinal tract from an E11.5 mouse showing the location of neural crest-derived cells (shown in grey; see Young and Newgreen, 2001). The regions labelled "a" (esophagus), "b" (midgut), and "c" (hindgut) were removed, suspended between a "V" cut into a piece of filter paper and grown in tissue culture medium for 4 days as described previously (Hearn et al., 1999). At the time of explantation, neural crest-derived cells (grey) were present throughout the explants of esophagus and midgut, whereas in the hindgut, they were only present at the rostral end of the explants (lower panels).

Slice explants grown on collagen gel

The esophagus and midgut from E11.5, E12.5, and E14.5 mice were dissected, cut into transverse sections about 0.5–1 mm thick, and the slices placed on collagen gels in a 5% CO_2 incubator at 37°C for 4 days. The collagen gels were made by restoring 4 mg/ml acidic collagen solution (Upstate, Parkville, Victoria, Australia) to normal osmolality with 5× DMEM and normal pH with 200 mM NaOH, on ice. This solution was diluted to 1 mg/ml collagen with tissue culture medium. GDNF, artemin, or neurturin was added to some collagen solutions before gelling to give a final concentration of 100 ng/ml.

Slice explants grown on filter paper

Transverse slices of esophagus and midgut from E11.5 mice were placed on 2.5 × 2.5 mm squares of filter papers. Each preparation was placed in a Terasaki well in 20 μl of tissue culture medium with or without 100 ng/ml GDNF, artemin, or neurturin and cultured in a 5% CO_2 incubator at 37°C for 4 days. The tissue culture medium was changed after 2 days.

Immunohistochemistry

Explants were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 4–24 hours. Explants grown on filter paper supports were left attached to the filter paper. After fixation, the explants were processed for immunohistochemistry using the antisera shown in Table 1. Whole-mount preparations of the gastrointestinal tract from the esophagus to the rectum from E12.5 and E14.5 mice were fixed for 2–4 hours in 4% paraformaldehyde in 0.1 M phosphate buffer or in Zamboni's fixative for 2–4 hours, and then processed for immunohistochemistry using GFRα1 and GFRα3 antisera (see Table 1).

Neuron counts within and outside suspended explants of gut

The total number of PGP9.5$^+$ neurons present on the filter paper supports outside the gut proper was counted, and the density of PGP9.5$^+$ neurons on the top surface of the gut explants was determined. To count the total number of PGP9.5$^+$ cells on the filter paper, images were taken on a fluorescence microscope using a 5× lens. A montage was made of the images using CorelDRAW10 and printed, and PGP9.5$^+$ neurons were counted from the print. In regions where the cells were clumped or overlapped, a 40× lens was used to count the neurons directly under the microscope. The total numbers of PGP9.5$^+$ neurons on the paper supports at both rostral and caudal ends were counted and added. Within the gut, the density of PGP9.5$^+$ neurons on the top surface only of the suspended explants was determined as the neurons along the bottom and sides of the gut explants could not be visualized clearly because of the tubular shape of the gut. The preparations were viewed on Bio-Rad MRC1000 or 1024 confocal microscopes (Bio-Rad, Richmond, CA) and images of PGP9.5$^+$ neurons in the suspended gut were taken by using a 20× objective lens. Images were printed and the number of neurons counted. The area of gut explant within which the neurons were counted was measured using Scion image software and then the density of neurons was calculated.

Table 1
Primary and secondary antibodies used

Primary antibodies			
	Host	Dilution	Source
PGP 9.5	Rabbit	1:2000	The Binding Site, Birmingham, UK
Neurofilament-M (145 kDa)	Rabbit	1:1000	Chemicon, Temecula, CA, USA
Hu	Human	1:2000	Fairman et al., 1995
NOS	Sheep	1:2000	Norris et al., 1995
Phox2b	Rabbit	1:700	Pattyn et al., 1997
GFRα1, GFRα3	Goat	1:25	R&D systems, Inc., Minneapolis, MN, USA

Secondary antibodies	
Species in which primary antibodies were raised	Secondary antibodies
Rabbit	Goat anti-rabbit Alexa 488 (1:250, Molecular Probes, Eugene, OR, USA)
Human	Donkey anti-human Texas Red (1:100, Jackson ImmunoResearch, West Grove, PA, USA)
Sheep or goat	Donkey anti-sheep FITC (1:100, Jackson ImmunoResearch), or Biotinylated donkey anti-sheep (1:100, Jackson ImmunoResearch) followed by streptavidin-Cy5 (1:100, Amersham)

Enteric neuron density within the esophagus of $Ret^{-/-}$, $Ret^{+/-}$, and $Ret^{+/+}$ mice

Mice in which cDNA encoding tau-EGFP-myc (TGM) had been inserted into the first coding exon of the receptor tyrosine kinase gene, *Ret*, were used (Enomoto et al., 2001). Mice heterozygous for the *Ret*-TGM mutation ($Ret^{TGM/+}$ mice) were mated. The genotype of adult and embryonic Ret^{TGM} mice was determined by PCR using the primers and conditions reported by Enomoto et al. (2001). The identity of E18.5 $Ret^{-/-}$ mice was confirmed by processing samples of duodenum from each embryo for NADPH diaphorase histochemistry (Ward et al., 1999). Samples of duodenum were fixed in 4% paraformaldehyde in 0.1M phosphate buffer for 1 h, washed, incubated in 10 mg β-NADPH, 2.5 mg nitroblue tetrazolium, and 20 μl Triton X in 10 ml 0.1 M Tris/HCl (pH 8.0) for 15–20 minutes at 37°C and then examined to determine if there were any NADPH diaphorase-stained neurons. E18.5 esophagus was processed for immunohistochemistry using antibodies to Hu (Table 1).

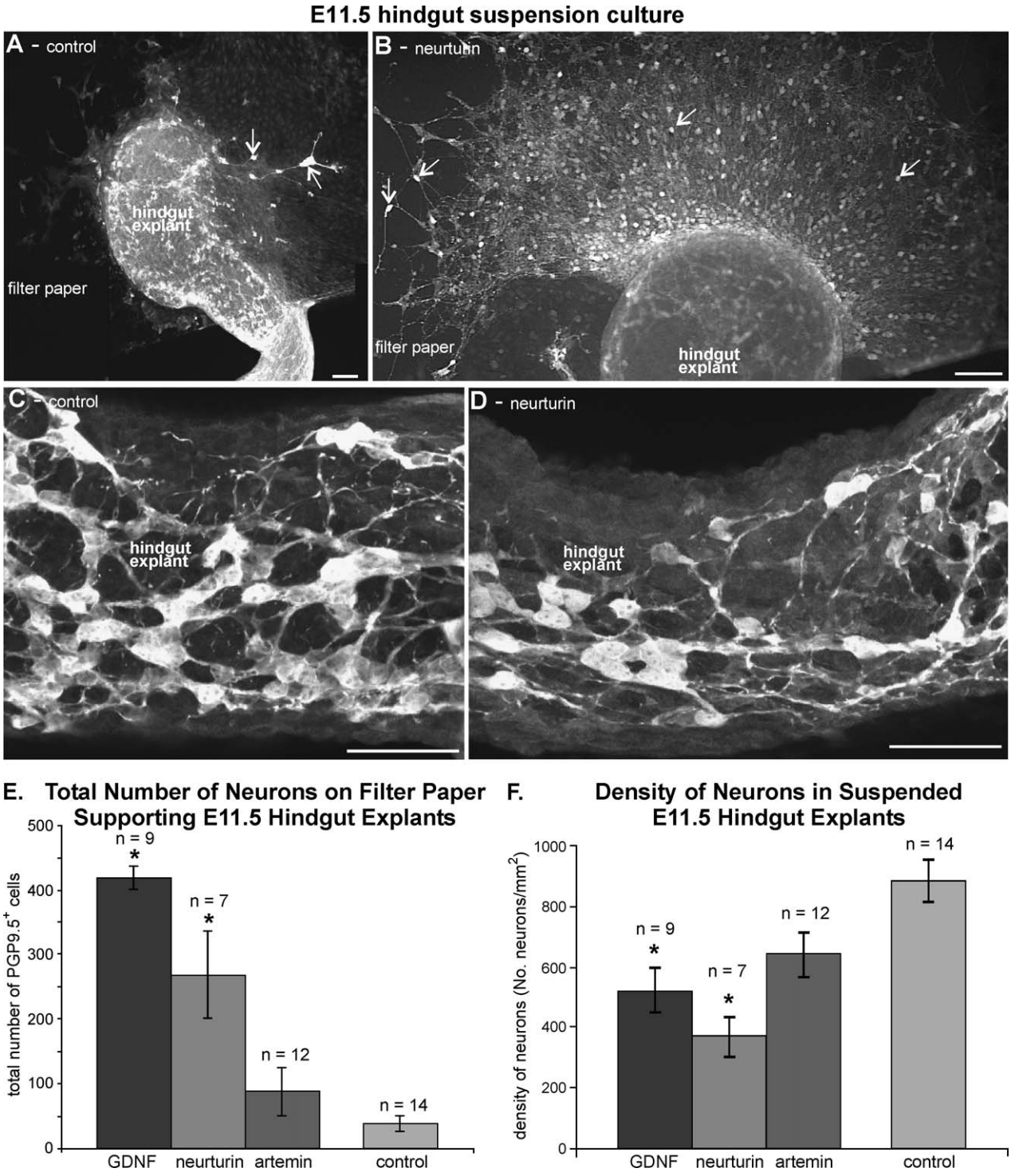

Fig. 2. Fluorescence micrographs of explants of E11.5 hindgut grown in control conditions (A, C) or in the presence of neurturin (B, D) for 4 days and then processed for immunohistochemistry using an antibody to the pan-neuronal marker, PGP 9.5. (A, B) The rostral ends of the explants. A small number of PGP9.5^{+} neurons (arrows) are present on the filter paper support under control conditions (A). (B) After culturing in the presence of neurturin, many PGP9.5^{+} neurons (arrows) are present on the filter paper support. (C, D) PGP9.5^{+} neurons within hindgut explants. The density of neurons is higher in the control explant (C) than in the explant grown in the presence of neurturin (D). Scale bars: 100 μm (A, B); 50 μm (C, D). Effects of GDNF, artemin, and neurturin on the total number of PGP9.5^{+} neurons on the filter paper supports outside of E11.5 hindgut explants (E), and the density of neurons within the explants (F). Data are means ± SEM. In the presence of GDNF or neurturin, both the total number of neurons on the filter paper and the densities of neurons within the suspended hindgut explants are significantly different from those in control conditions, but explants grown in the presence of artemin do not show a significant difference from controls in either the number of neurons on the filter paper or the density of neurons within the explant (ANOVA followed by a Tukey test).

The density of Hu^+ cells on the top surface of each esophagus was determined using the same technique as that used to determine the density of neurons in suspension cultures (see above) except that images were taken on a conventional fluorescence microscope using a 10× objective lens. In addition, the proportion of Hu^+ neurons that showed NOS immunostaining was determined in some E18.5 preparations. The entire gastrointestinal tract from E11.5 *Ret* transgenic mice was processed for immunohistochemistry using an antibody to Phox2b, to ascertain the entire enteric neural crest cell population.

Statistical analysis

Student's two-tailed unpaired *t* tests or repeated measures ANOVA followed by Tukey post hoc tests were performed where appropriate. A probability of less than 0.05 ($P < 0.05$) was considered significant throughout.

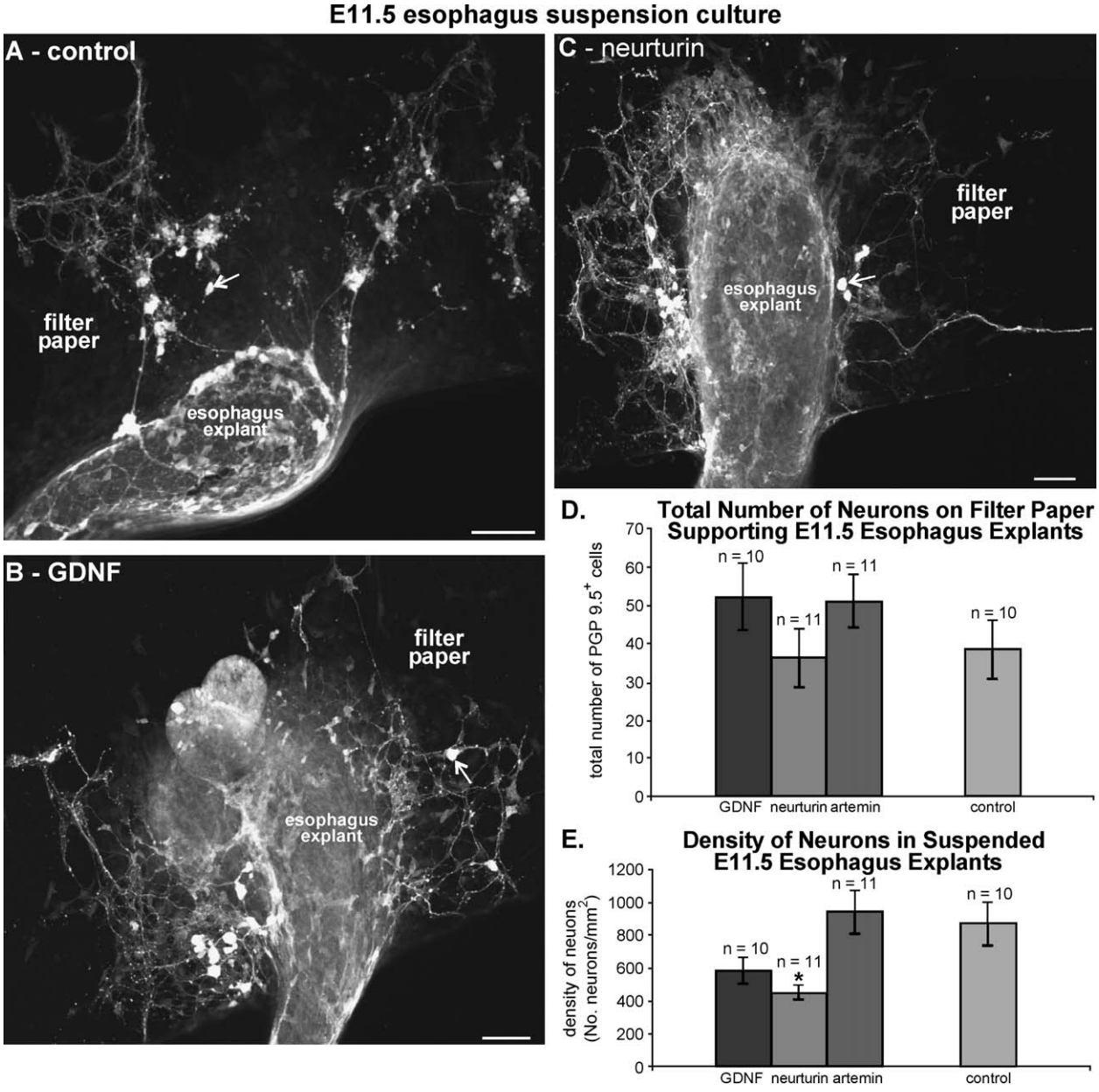

Fig. 3. The rostral ends of explants of E11.5 esophagus grown in control conditions (A) or in the presence of GDNF (B) or neurturin (C) for 4 days and then processed for immunohistochemistry using an antibody to the pan-neuronal marker, PGP9.5. There are few $PGP9.5^+$ neurons (arrows) on the filter paper supports under all three conditions. Scale bars: 100 μm. Effects of GDNF, artemin, and neurturin on the total number of neurons on the filter paper supports outside of E11.5 esophagus explants (D) and on the density of neurons within the explants (E). Data are means ± SEM. There was no significant difference between the four groups in the number of neurons on the filter paper supports, but the density of neurons within the explants cultured in the presence of neurturin was significantly lower than that in controls (ANOVA followed by a Tukey test). The densities of neurons within explants grown in the presence of GDNF or artemin were not significantly different from controls.

Results

Neural cell migration response induced by GDNF, artemin, or neurturin in different regions of embryonic mouse gut grown in suspension organ culture

Hindgut explants

Explants of caecum and post-caecal hindgut from E11.5 mice were grown in suspension organ culture on filter paper supports in control culture medium or with added GDNF, artemin, or neurturin (100 ng/ml). At the time of explantation, neural crest cells are present in the most rostral end of each hindgut explant only (caecum and rostral-most post-caecal hindgut) (Fig. 1; Young and Newgreen, 2001). After 4 days in culture, the explants were fixed and processed for PGP9.5 immunohistochemistry to reveal neurons. The density of PGP9.5$^+$ cells within the explants, and the total number of PGP9.5$^+$ cells on the filter paper supports, were determined.

Under control conditions, many PGP9.5$^+$ neurons were present throughout the gut explants (Figs. 2C,F) and only a small number of neurons was found on the filter paper (Figs. 2A,E), mostly at the rostral end of the explants. Thus, neural crest cells had migrated through the explants and differentiated into neurons. In the presence of artemin, the density of neurons within the explants and the number of neurons on the filter paper supports were not significantly different from controls (ANOVA; Figs. 2E,F). However, in the presence of GDNF or neurturin, the density of PGP9.5$^+$ neurons within the suspended gut explants was significantly lower than that in control explants (Figs. 2D,F), and the number of PGP9.5$^+$ neurons on the filter paper supports was significantly higher than in controls (ANOVAs; Figs. 2B,E). These data confirm a previous study that showed that GDNF in the culture medium can promote the migration of neural crest cells out of suspended hindgut explants in vitro (Young et al., 2001). The results also show that neurturin, but not artemin, has similar effects to GDNF on the migratory behavior of neural crest cells in the hindgut.

Esophagus explants

Explants of E11.5 esophagus (rostral foregut) were grown suspended on filter papers supports. Note that explants of E11.5 esophagus differ from E11.5 hindgut explants in that neural crest-derived cells are present throughout the entire rostro-caudal extent of the explant at the time of explantation (Fig. 1). After 4 days in culture, the total number of PGP9.5$^+$ neurons present on the filter paper supports in the presence of GDNF, artemin, or neurturin was not significantly different from controls (ANOVA; Fig. 3). The density of neurons within explants of esophagus grown in the presence of GDNF or artemin was also not significantly different from controls, but the density of neurons within esophagus explants grown in the presence of neurturin was significantly lower than that in control explants (ANOVA; Fig. 3E). Suspension cultures of E10.5 esophagus were also attempted to examine if GDNF family members could induce migration from younger suspended explants of esophagus. At E10.5, the esophagus is short and very fragile, and we were unable to grow them successfully as suspended explants.

Midgut explants

Segments of E11.5 and E12.5 midgut were grown in suspension cultures with or without added GDNF. The colonization of the midgut by neural crest-derived cells begins around E10.5, and by E11.5, the entire midgut has been colonized (see Young and Newgreen, 2001). Thus, like the esophagus from E11.5 mice, these segments of midgut have neural crest-derived cells throughout the entire segment at the time of explantation. Data showing the effect of GDNF on PGP9.5$^+$ neuron number outside of, and within, suspended explants of E11.5 and E12.5 midgut are shown in Fig. 4. At both E11.5 and E12.5, the number of neurons on the filter paper supports of E11.5 and E12.5 midgut explants grown in the presence of GDNF was significantly greater than controls (unpaired *t* test; Fig. 4A). However, the effect

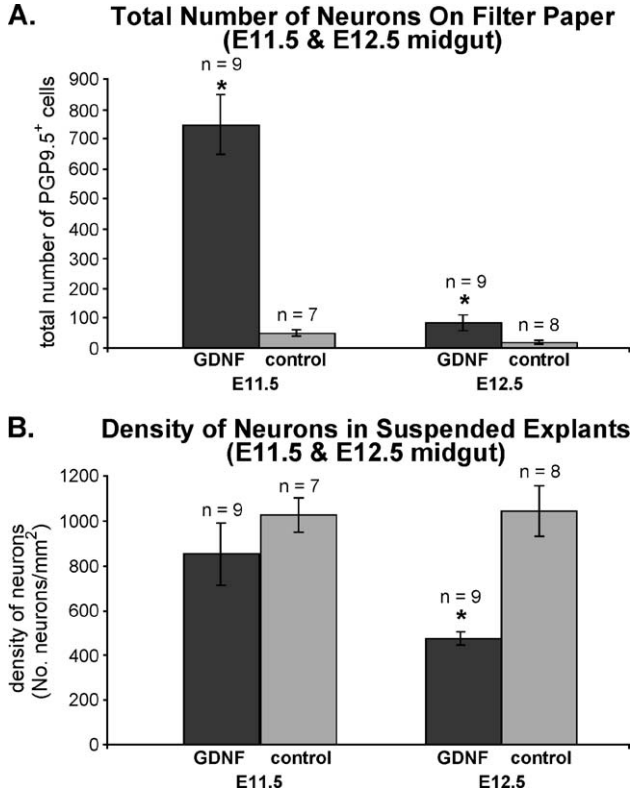

Fig. 4. Effects of GDNF on the total number of PGP9.5$^+$ neurons on the filter paper supports (A) and density of neurons within explants (B) of explants of E11.5 and E12.5 midgut. Data are means ± SEM. (A) At both E11.5 and E12.5, the total numbers of neurons on the filter paper supports of midgut explants grown in the presence of GDNF were significantly higher than those grown in control conditions (unpaired *t* test). (B) At E12.5, the density of neurons within the control midgut explants was significantly higher than the explants grown in the presence of GDNF (unpaired *t* test), but at E11.5, there was no significant difference between the two groups (unpaired *t* test).

of GDNF on the number of neurons on the filter paper declined dramatically with age. In the presence of GDNF, the density of neurons within the gut explants was significantly lower than in the controls at E12.5 (unpaired *t* test), but not at E11.5 (unpaired *t* test; Fig. 4B). This difference may reflect differing effects of GDNF on proliferation and/or differentiation at different ages. These data demonstrate that even when neural crest cells are present throughout midgut explants at the time of explantation, GDNF is capable of inducing an increase in the number of neurons on the filter paper supports, but that the effect of GDNF on the migratory behaviour of neural crest-derived cells in the midgut declines with age.

Neural crest-derived cell migration and neurite outgrowth from transverse slices of esophagus and midgut grown on collagen gels with or without GDNF, artemin, or neurturin

E11.5 esophagus

Transverse slices of esophagus were grown on collagen gels, with or without 100 ng/ml GDNF, artemin, or neurturin within the gel. After 4 days in culture, the explants

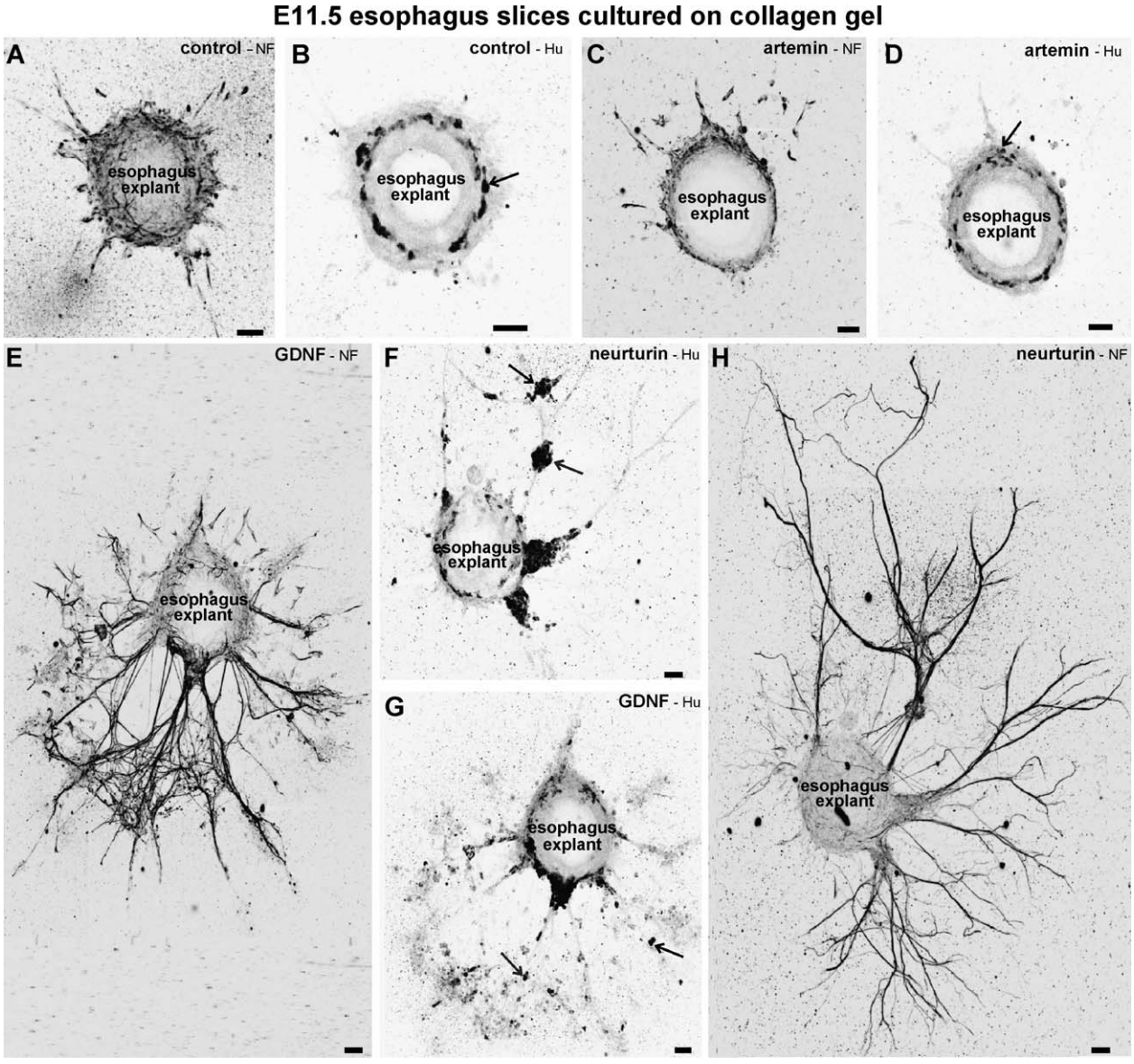

Fig. 5. Inverted fluorescence images of transverse slices of E11.5 esophagus grown on collagen gels with or without GDNF, artemin, or neurturin in the gels and then processed for immunohistochemistry using antibodies to neurofilament (NF, to label neurites) and Hu (to label nerve cell bodies). In the controls and the presence of artemin, there were no or few neurites extending out of the explants (A, C), and most Hu^+ neurons (arrows) were present within the external muscle layer of the explant (B, D). In the presence of GDNF and neurturin, many neurites extended from the explant into the collagen gel (E, H) and many Hu^+ nerve cell bodies (arrows) were found outside the esophagus explants (F, G). Most of the nerve cell bodies outside of the explants occurred in clumps. Scale bars: 50 μm.

were immunostained for Hu to reveal neural cell bodies and for neurofilament to reveal neurites. In control conditions, or with artemin added to the gels, many Hu^+ cells were present within the explant, and they formed a discrete layer in the outer part of the mesenchyme (Figs. 5B,D). A small number of Hu^+ cells was found close to, but outside, the explants in a few specimens (2/12 controls and 1/12 with artemin present). In control or artemin-treated explants, there were either no neurites, or only sparse neurites, that extended away from the explants (Figs. 5A,C). In the presence of GDNF or neurturin, many Hu^+ cells were observed on the gel outside of the explants (Figs. 5F,G). The Hu^+ cells outside the explants were mainly found in clumps (Figs. 5F,G). Most esophagus explants grown on gels containing GDNF or neurturin also possessed many bundles of neurofilament$^+$ neurites that extended away from the explants (8/12 with GDNF present in the gel, 10/12 with neurturin; Figs. 5E,H). The clumps of Hu^+ cells were usually found associated with bundles of neurites. Hence, in contrast to suspended explants of E11.5 esophagus, GDNF and neurturin induced a migratory and neurite outgrowth response from transverse slices of E11.5 esophagus grown on collagen gels.

E12.5 and E14.5 esophagus

E12.5 ($n = 12$) and E14.5 ($n = 6$) esophageal slices grown in the presence of GDNF did not show a migratory response (i.e., no Hu^+ cells were present outside of the explants). A neurite outgrowth response to GDNF was seen in only 42% (5/12) E12.5 explants and 33% (2/6) of E14.5 explants exposed to GDNF, but in these explants, only short neurofilament$^+$ neurites extended from the explants (Fig. 6B). However, strong neurite outgrowth responses were induced by neurturin from the E12.5 ($n = 6$) and E14.5 ($n = 6$) esophagus explants (Fig. 6D), although Hu^+ cell bodies were only rarely (1/6) observed outside the explants (Fig. 6C).

E11.5 midgut

Transverse sections of E11.5 midgut were grown on collagen gels under control conditions or with GDNF, neurturin, or artemin added to the gel. In control and artemin-treated explants, Hu^+ cells were observed within

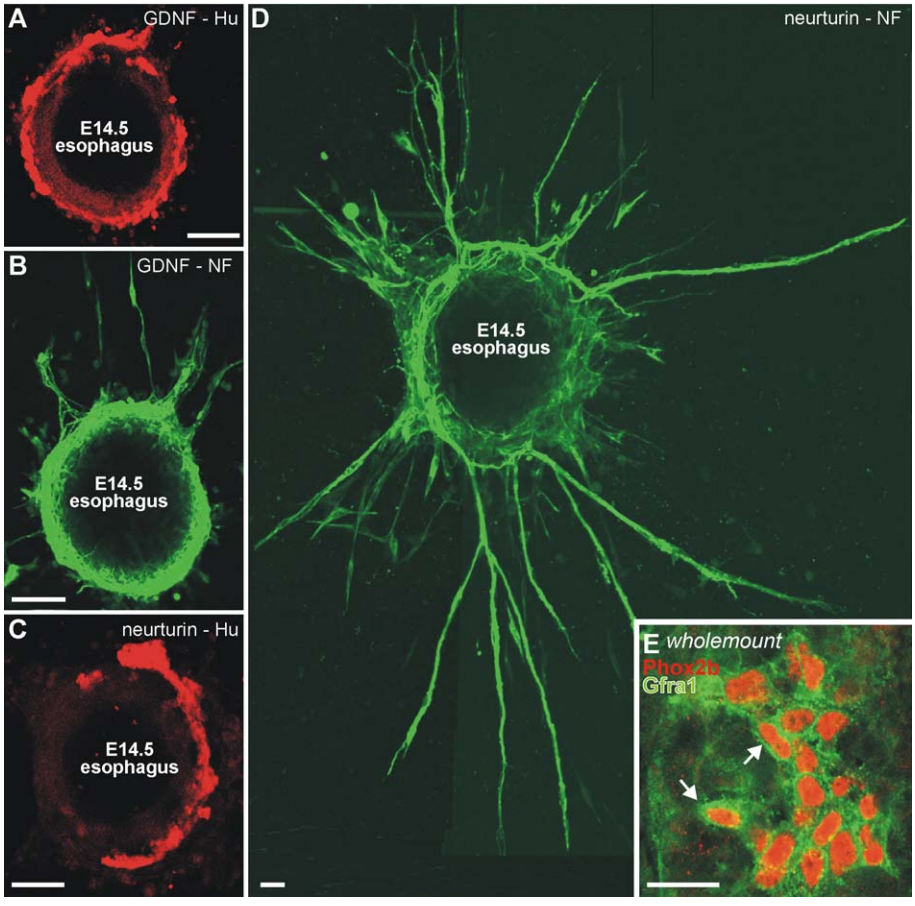

Fig. 6. (A–D) Fluorescence images of transverse slices of E14.5 esophagus grown on collagen gels with GDNF or neurturin in the gels and then processed for immunohistochemistry using antibodies to neurofilament (NF) and Hu. In the presence of GDNF (A, B), there were few neurites extending from the esophagus explants and rare Hu^+ neurons outside of the explants. However, in the presence of neurturin, many neurites extended onto the collagen gel outside of the explants (D) but few Hu^+ cell bodies were present outside of the explant (C). (E) Whole-mount (uncultured) preparation of esophagus from an E14.5 mouse processed for immunohistochemistry using antisera to Phox2b (red, to label all neural crest cells) and GFRα1 (green). All Phox2b$^+$ cells show strong GFRα1 immunostaining, predominantly on their cell membranes (arrows). Scale bars A–D: 100 μm, E: 25 μm.

all of the explants, and a small number of Hu^+ cells was observed outside of the explants in some experiments (4/12 of control and 3/12 of artemin-treated) (Figs. 7B,D). Sparse neurites extended from some of the explants in 7/12 of control (Fig. 7A) and 3/12 of artemin-treated (Fig. 7C). In contrast, extensive neurite outgrowth was observed extending from all midgut slices grown on collagen gels containing GDNF ($n = 12$) or neurturin ($n = 12$) (Figs. 7G,E), and some of the neurites extended for over 1 mm away from the explants. In the presence of GDNF or neurturin, many Hu^+ cells were observed both within the midgut slice explants and in clumps associated with neurites outside of the explants (Figs. 7H,F).

E12.5 and E14.5 midgut

In the presence of GDNF or neurturin, Hu^+ cells were present outside of the midgut explants and neurites extended from the explants onto the collagen gel at both E12.5 ($n = 12$) and E14.5 ($n = 6$) (data not shown).

Neural crest-derived cell migration and neurite outgrowth from transverse slices of E11.5 esophagus and midgut grown on filter paper with or without GDNF, artemin, or neurturin present in the culture medium

The experiments in which suspended tubes of E11.5 esophagus were grown on filter paper supports suggested

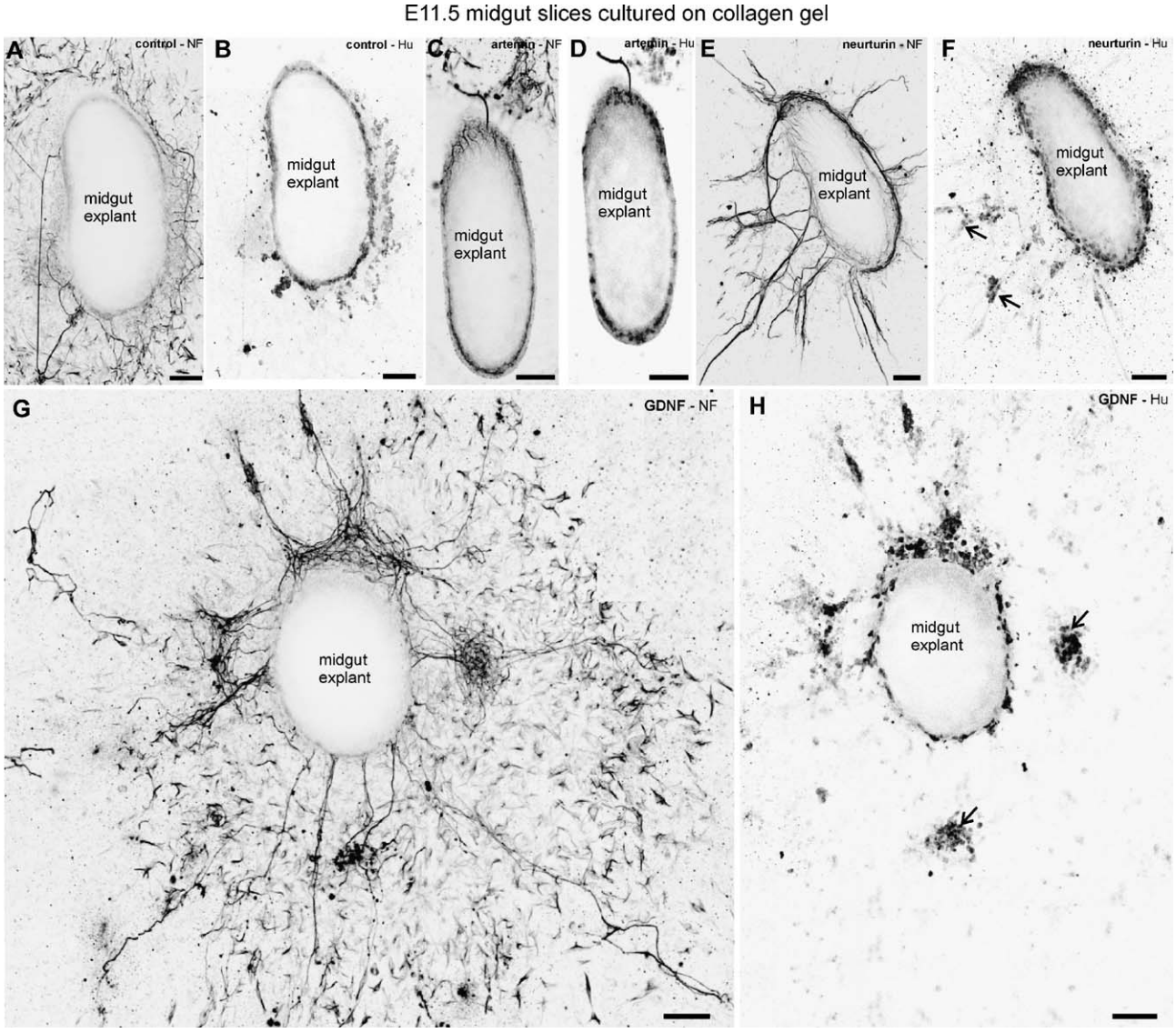

Fig. 7. Inverted fluorescence images of transverse slices of E11.5 midgut grown on collagen gels with or without GDNF, artemin, or neurturin in the gels and then processed for immunohistochemistry using antibodies to neurofilament (NF) and Hu. Under control conditions (A, B) or in the presence of artemin (C, D), there were few neurites extending from the midgut explants and few Hu^+ nerve cell bodies outside of the explants. However, many neurites and neurons (arrows) were found outside of the midgut explants in the presence of GDNF (G, H) and neurturin (E, F). Most of the nerve cell bodies outside of the explants occurred in clumps. Scale bars: 100 μm.

that GDNF and neurturin do not promote migration of esophageal neural cells, whereas the experiments in which transverse slices of E11.5 esophagus were grown on collagen gels containing GDNF or neurturin suggested that both these factors induce migration of neural cells from the esophagus. To determine whether the differences were due to differences in the size and shape of the explants (whole tubes versus transverse slices) or difference in the substrate (filter paper versus collagen gel), transverse slices of E11.5 esophagus and midgut were attached to small squares of filter paper and grown in culture medium for 4 days.

Esophagus

Similar results were found in control conditions and in the presence of artemin. Many Hu^+ cells were present within the outer mesenchyme of the esophagus slice explants, but there were few or no Hu^+ cells outside of the explants (Figs. 8A,B) and few or no neurites extending from the explant onto the filter paper. In the presence of GDNF and neurturin, some neurites extended from the explants onto the filter paper, but the number of neurites appeared to be less than that observed on collagen gels. There were very few Hu^+ cells present within the explants, but many Hu^+ cells outside of the explants in the GDNF and neurturin-treated explants (Figs. 8C,D). However, in contrast to the slice explants grown on collagen gel, many of the Hu^+ cells outside of the explants were not found in clumps (Figs. 8C,D). Hence, the migratory response induced by GDNF and neurturin from transverse slices of E11.5 esophagus onto a filter paper substrate is similar to that seen in collagen gels.

Midgut

Slices of midgut grown on filter paper responded to GDNF family members in a similar manner to that observed when they were grown on collagen gels (data not shown). In control conditions, many Hu^+ cells were present within the wall of the midgut slices, but no or few neurites extended from the explants onto the filter paper. Some control explants had a small number of Hu^+ cells outside, but close to, the explant. In the presence of artemin, a few neurites (<10) were observed extending from some explants (3/6) and a small number of Hu^+ cells were associated with these neurites. Many Hu^+ cells were present within the explant wall of artemin-treated explants. In the presence of GDNF

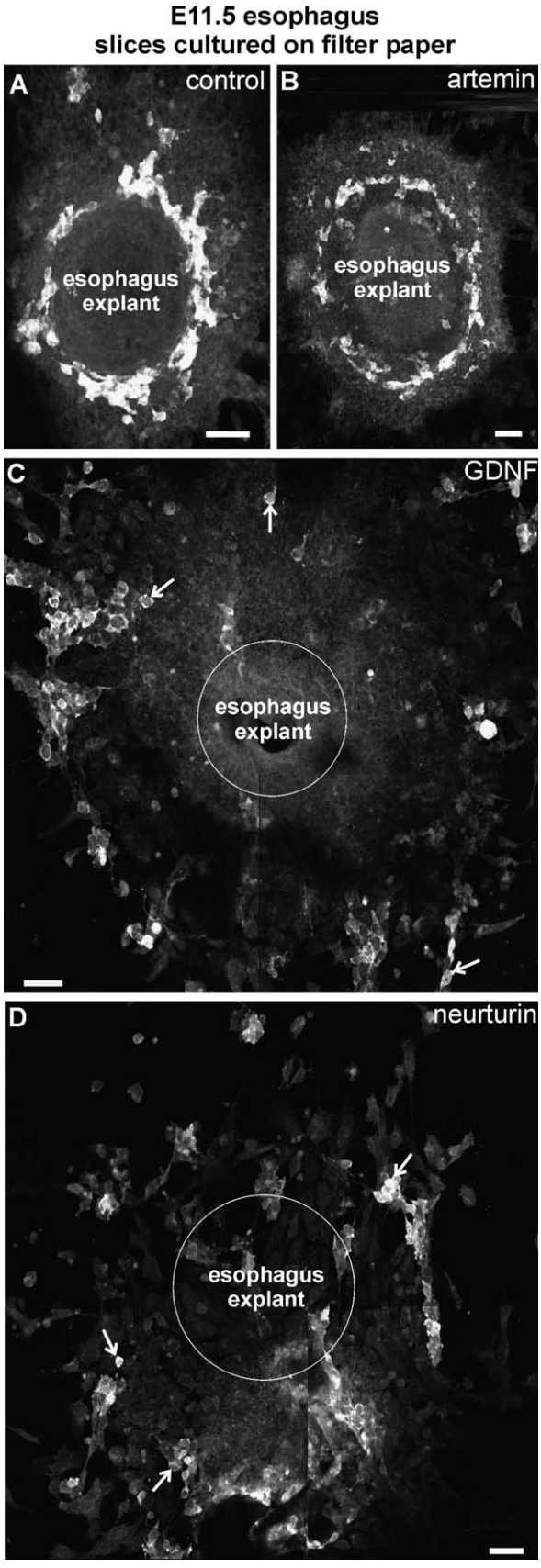

Fig. 8. Fluorescence images of transverse slices of E11.5 esophagus grown on filter paper in control condition (A), or with artemin (B), GDNF (C), or neurturin (D) added to the culture medium for 4 days, and then processed for immunohistochemistry using an antibody to Hu to label nerve cell bodies. Under control conditions (A) or in the presence of artemin (B), Hu^+ cell bodies were present in the external muscle layer of the slices and few were present outside of the explants. In the presence of GDNF (C) or neurturin (D), many Hu^+ neurons (arrows) were present on the filter paper, outside of the explants, and there were very few neurons within the explants (whose locations are indicated with a dotted line). Scale bars: 50 μm.

(n = 6) or neurturin (n = 6), many Hu^+ cells were present both within and outside of midgut slice explants and many neurites extended from the explant onto the filter paper. The migrating neurons were closely associated with nerve fibres.

GFRα1 and GFRα3 immunostaining

GFRα1

The experiments described above showed that the GDNF-induced neurite outgrowth and migratory responses of neural crest-derived cells in the esophagus declined with age, whereas the response of midgut neural cells to GDNF was maintained until at least E14.5. To examine whether the decline in responsiveness of esophageal neural cells is due to a down-regulation of GFRα1, we examined the presence of GFRα1 immunostaining in whole-mount preparations of esophagus and midgut using an antiserum that has previously been shown to be specific for GFRα1 (Golden et al., 2003). At E14.5, neural crest cells (identified by Phox2b immunostaining) in both the esophagus (Fig. 6E) and midgut showed strong GFRα1 immunostaining, and there was no obvious difference in the levels of immunostaining between cells in the two different gut regions. Thus, the decline in responsiveness of cells in the esophagus does not appear to be due to a down-regulation of GFRα1.

GFRα3

Unlike sympathetic precursors (Honma et al., 2002), we did not detect any migratory or neurite outgrowth response to artemin in any gut region at any developmental stage examined. To determine whether the lack of effect of artemin on enteric crest-derived cells is due to the lack of expression of GFRα3, we processed whole-mount preparations of sympathetic ganglia, midgut, and esophagus from E12.5 and E14.5 mice for GFRα3 immunostaining. Although GFRα3 immunostaining was observed in sympathetic ganglia, we did not observe any detectable GFRα3 immunostaining in crest-derived cells in either the esophagus or midgut (data not shown).

Comparison of neural crest-derived cell density within the esophagus of E11.5 and E18.5 $Ret^{+/+}$, $Ret^{+/-}$, and $Ret^{-/-}$ mice

The experiments described above showed that neural crest-derived cells in the esophagus show similar neural

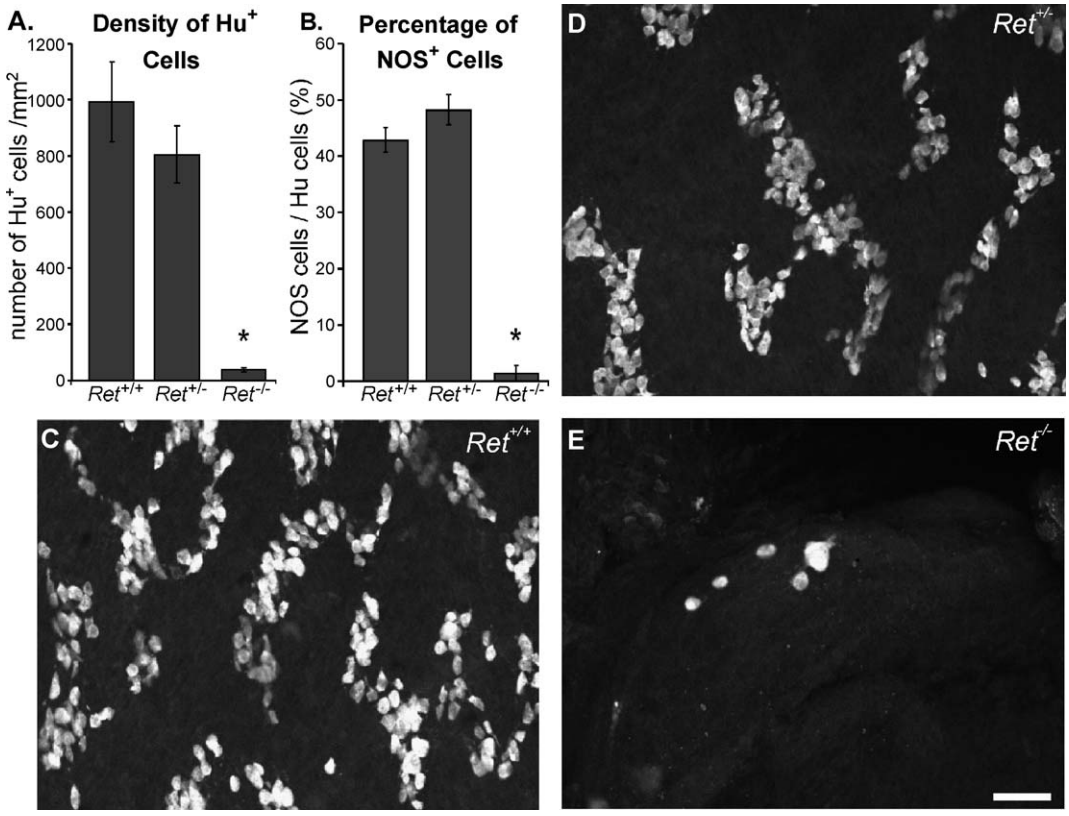

Fig. 9. Density of Hu^+ (A) and NOS^+ cells as a percentage of Hu^+ cells (B) within the esophagus of E18.5 $Ret^{+/+}$, $Ret^{+/-}$, and $Ret^{-/-}$ mice. Data are means ± SEM. The density of Hu^+ cells and the percentage of Hu^+ cells that express NOS within the esophagus of $Ret^{-/-}$ mice are significantly lower than that in wild-type and $Ret^{+/-}$ mice (ANOVAs). There was no significant difference between the wild-type mice and $Ret^{+/-}$ mice. (C–E) Micrographs of whole mounts of esophagus stained using Hu antisera. Many Hu^+ cells are present within the esophagus of $Ret^{+/+}$ (C) and $Ret^{+/-}$ (D) mice. (E) In the esophagus of $Ret^{-/-}$ mice, very few Hu^+ cells were present. Scale bar: 50 μm (applies to C–E).

migration responses to GDNF and neurturin to cells in the midgut and hindgut. A previous study reported that enteric neurons are present in the esophagus of *Ret*$^{-/-}$ mice (Durbec et al., 1996). However, the number of neurons present in the esophagus of mice lacking members of the GDNF-signalling pathway has not been examined quantitatively.

E18.5 esophagus

We examined the density of all enteric neurons in the E18.5 esophagus using Hu antisera. The identity of *Ret*$^{-/-}$ mice determined by PCR was confirmed by NADPH diaphorase staining of samples of duodenum (Ward et al., 1999). The density of Hu^{+} cells on the top surface of the entire esophagus was determined in five *Ret*$^{-/-}$, five *Ret*$^{+/-}$, and five *Ret*$^{+/+}$ mice. The density of Hu^{+} cells in the esophagus of E18.5 *Ret*$^{-/-}$ mice was significantly lower than that in the esophagus of both wild-type and *Ret*$^{+/-}$ mice (ANOVA; Fig. 9), and was only about 4% that of wild-type and heterozygous mice. The density of Hu^{+} cells in the esophagus of *Ret*$^{+/-}$ mice was not significantly different from wild-type mice (ANOVA; Fig. 9). A major sub-class of neurons in the mouse esophagus expresses nitric oxide synthase (NOS) (Sang and Young, 1997; Worl et al., 2002). The proportion of Hu^{+} cells that showed NOS immunostaining was examined in three *Ret*$^{-/-}$, three *Ret*$^{+/-}$, and three *Ret*$^{+/+}$ mice. In wild-type and heterozygous mice, around 40% of Hu^{+} cells were NOS^{+} (Fig. 9B). In contrast, of 121 Hu^{+} cells examined in the esophagus of *Ret*$^{-/-}$ mice, only 1 cell showed NOS immunostaining.

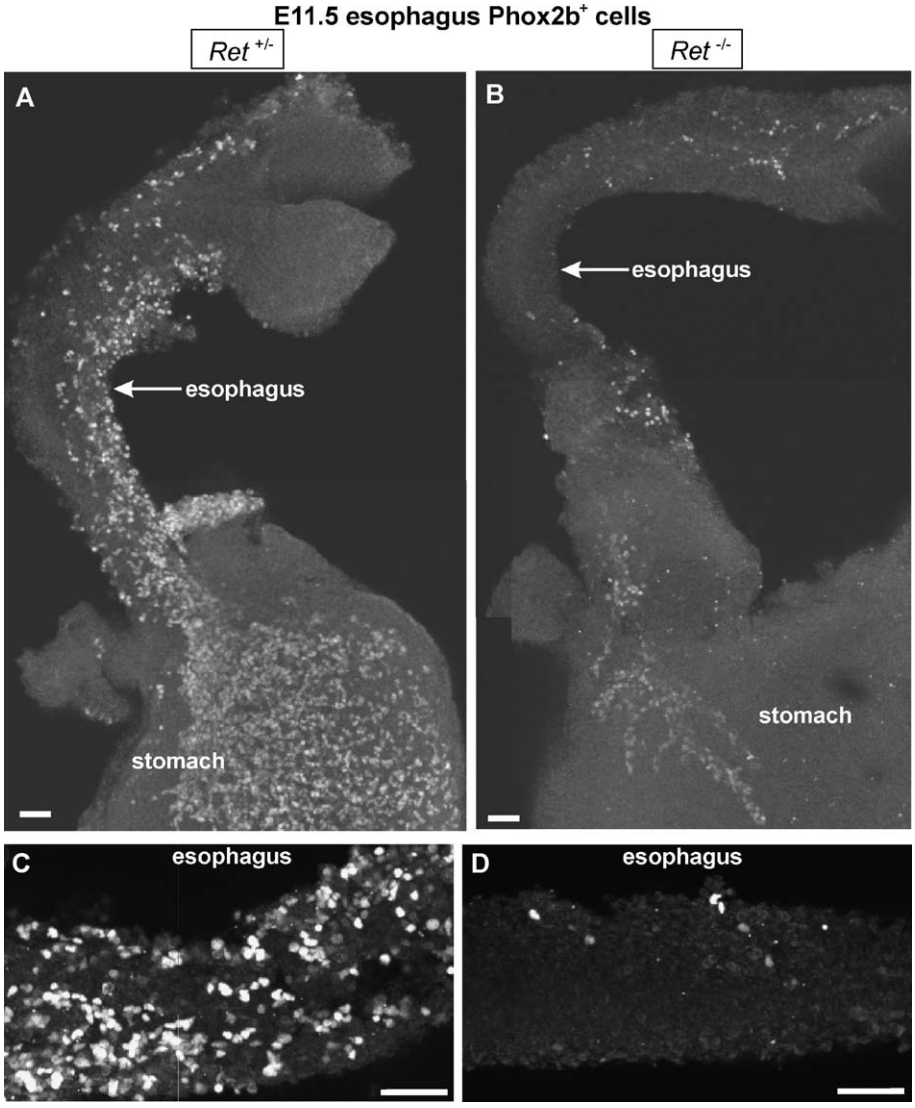

Fig. 10. Low (A, B) and higher (C, D) magnification images showing the distribution of Phox2b^{+} neural crest-derived cells within the esophagus of E11.5 *Ret*$^{+/-}$ (A, C) and *Ret*$^{-/-}$ (B, D) mice. Many Phox2b^{+} cells were present along the entire esophagus of the *Ret*$^{+/-}$ mouse (A, C). However, only a small number of Phox2b^{+} cells were present within the wall of esophagus of *Ret*$^{-/-}$ mice (B, D). Most Phox2b^{+} cells present in the esophagus of E11.5 *Ret*$^{-/-}$ mice were in the rostral region. There were also some Phox2b^{+} cells in the stomach of *Ret*$^{-/-}$ mice (B). Scale bars: 50 μm.

E11.5 esophagus

To determine whether the decrease in the density of neurons in the esophagus of E18.5 mice is due to failure of the neural crest cells to colonize the esophagus or a failure to survive and differentiate, we examined the immunolocalization of Phox2b in the E11.5 gastrointestinal tract. Phox2b appears to be expressed by all neural crest-derived cells within the gastrointestinal tract (Young et al., 2003). In the esophagus of E11.5 wild-type ($n = 7$) and $Ret^{+/-}$ ($n = 6$) embryos, many Phox2b$^+$ cells were present along the whole esophagus. However, in the esophagus of homozygous mutant E11.5 embryos ($n = 7$), only sparse Phox2b$^+$ cells were seen within the wall of esophagus (Fig. 10). Most Phox2b$^+$ cells present in the esophagus of E11.5 $Ret^{-/-}$ mice were in the rostral esophagus (data not shown). In E11.5 homozygous mutant mice, Phox2b$^+$ cells were observed outside of the esophagus along the pathway of the vagus nerve, and there were also Phox2b$^+$ cells in the stomach, but in vastly reduced numbers compared to wild-type or $Ret^{+/-}$ embryos (Fig. 10).

Discussion

The results of this study show that (i) neurturin, like GDNF, is chemoattractive to enteric neural crest-derived cells and also induces neurite outgrowth; (ii) GDNF and neurturin promote the migration of crest-derived cells from the esophagus as well as the midgut and hindgut; (iii) artemin has no detectable effect on migration or neurite outgrowth in any region examined; (iv) the migratory responses induced by GDNF or neurturin decline with age; and (v) the development of esophageal neurons is largely Ret-dependent.

Neurturin has similar effects to GDNF on migration and neurite outgrowth

A previous study has shown that GDNF and neurturin have similar effects on the survival, proliferation, and differentiation of neural crest-derived cells isolated from the gut (Taraviras et al., 1999). Our study showed that exogenous neurturin also has similar effects to GDNF on the migration of enteric crest-derived cells, and both neurturin and GDNF were chemoattractive to crest-derived cells in esophagus, mid-, and hindgut explants. The current study also showed that both neurturin and GDNF induced neurite outgrowth from explants of embryonic gut.

Neurturin is not essential for the migration of neural precursors into and along the developing gut in vivo. Mice lacking neurturin or its ligand binding molecule, GFRα2, possess an enteric nervous system along the entire length of the gastrointestinal tract (Gianino et al., 2003; Heuckeroth et al., 1999; Rossi et al., 1999, 2003). Furthermore, although there is some expression of neurturin in the mucosa of E12 mouse gut, neurturin expression is not detected in the gut mesenchyme (through which neural crest cells migrate) until E14 (Golden et al., 1999); this is after the colonization of the embryonic mouse gut by crest-derived cells is complete (Kapur et al., 1992). In contrast, GDNF signalling is necessary for neural cell migration in the intestine (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Tomac et al., 2000), and GDNF is expressed by the gut mesenchyme before the entry of neural crest-derived cells (Natarajan et al., 2002). However, mice lacking neurturin or GFRα2, which forms part of the receptor complex for neurturin, have fewer nerve fibres in the circular muscle (Heuckeroth et al., 1999; Rossi et al., 1999, 2003), where neurturin is normally primarily expressed. Thus, neurturin seems to be important for inducing axon outgrowth into the circular muscle layer.

Studies using cell lines have shown that neurturin can activate Ret by binding to either GFRα1 or GFRα2 (Creedon et al., 1997; Jing et al., 1997). Hence, the responses induced by neurturin described in the current study could be due to neurturin binding to either GFRα1 or GFRα2, or to both.

Neural cell migration and axon outgrowth have many features in common (Rakic, 1999), and it is likely that the response (migration or axon extension) induced by activation of Ret by GDNF or neurturin will depend on the age and state of differentiation of the enteric crest-derived cells. Ret activation in younger or undifferentiated enteric crest-derived cells may bias the response toward migration, whereas Ret activation in older, post-migratory cells may bias the response toward neuronal differentiation and axon outgrowth (Hearn et al., 1998; Wu et al., 1999).

Enteric neural crest-derived cells did not show a migratory or neurite outgrowth response to artemin

Artemin appears to play a role in migration and neurite outgrowth during the development of the sympathetic nervous system (Enomoto et al., 2001; Honma et al., 2002; Nishino et al., 1999). The only gastrointestinal region that expresses artemin is the esophagus (Enomoto et al., 2001). Despite the expression of artemin in the esophagus, enteric neural crest-derived cells in both the esophagus and the intestine failed to show a migratory or neurite outgrowth response to artemin at the ages examined in this study. However, we also showed that, unlike sympathetic neuron precursors, enteric neural crest-derived cells do not appear to express GFRα3, which would account for their lack of response to artemin.

The migratory response induced by GDNF and neurturin declines with age

Our data showed that the GDNF-induced migratory responses in both the esophagus and midgut declined with age because (a) transverse slices of E11.5 esophagus grown on collagen gel showed a strong migratory response to

GDNF and neurturin, but E12.5 and E14.5 esophageal slices showed little or no migratory response; and (b) the chemoattractive response to GDNF of crest-derived cells in suspended explants of E12.5 midgut was dramatically lower than that of E11.5 suspended midgut explants (see Fig. 4). The earlier age-dependent decline in the esophagus compared to the midgut parallels the earlier colonization of the esophagus by neural crest cells. In addition, our study showed that the GDNF-induced neurite outgrowth response from the esophagus also declined with age. In fetal rats, the proliferative responses and neuronal differentiation induced by GDNF or neurturin also decline between E12.5 and E14.5/E15.5 (Chalazonitis et al., 1998; Taraviras et al., 1999).

The decline in responsiveness to GDNF does not appear to be due to a down-regulation of GFRα1, as E14.5 esophageal neural cells showed strong GFRα1 immunostaining, but did not show GDNF-induced migratory or neurite-outgrowth responses. Thus, the age-dependent decline in responsiveness appears to be due to changes downstream of GFRα1. It is unclear whether the decline in the GDNF-induced migratory responses with age reflects an age-dependent decrease in migratory ability, or other factors such as an increase in cell–cell adhesion, which may prevent cells from migrating.

Although GDNF induced a migratory response from transverse slices of E11.5 esophagus grown on collagen gels or on filter paper, we did not detect a migratory response from suspended explants of E11.5 esophagus. We believe this is because the crest-derived cells have to migrate further to escape the tubular suspended explants than they do to escape the transverse slices. Because the esophagus of E10.5 mice is fragile, we could not reliably set them up as suspended explants for quantitative analysis.

There was also a decline with age of the migratory response of neural cells in the esophagus induced by neurturin. However, unlike GDNF, there was still a robust neurite outgrowth response from the esophagus and midgut induced by neurturin at E14.5, the oldest age examined. Because of the thickness of the gut wall, it was not possible to establish suspension or slice cultures of gut from older fetuses. However, as neurturin appears to be involved in inducing axon outgrowth into the circular muscle, and as the innervation of the circular muscle continues to develop after birth (Young et al., 1998), it appears likely that the neurites of enteric neurons will be responsive to neurturin postnatally.

GDNF and neurturin promote the migration of crest-derived cells from the esophagus as well as the mid- and hindgut

The rostrocaudal origin of neural crest cells that give rise to esophageal neurons may vary between birds and rodent (see Newgreen and Young, 2002). In mice, a DiI-tracing study showed that esophageal neurons arise from neural crest cells that emigrate adjacent to somites 6–7, whereas intestinal neurons arise from neural crest cells that emigrate from the hindbrain next to the more rostral somites (Durbec et al., 1996). Neural crest cells adjacent to somites 6–7 also give rise to dorsal root ganglia, and thus this level of the neural axis can also be classified as "truncal" (Durbec et al., 1996). In contrast, Burns et al. (2000) using cell labelling in birds suggest that all esophageal neuron arise from the rostral limit of the vagal region, not from the caudal or truncal limit.

Because (a) esophageal neurons in embryonic mice arise from a slightly different rostrocaudal level of the neural axis from intestinal neurons (Durbec et al., 1996), (b) neurons have been reported to be present in the esophagus and stomach but absent elsewhere in the gastrointestinal tract in *Ret*$^{-/-}$ mice (Durbec et al., 1996), and (c) neurons are absent from the esophagus but present elsewhere in the gastrointestinal tract of *Mash1*$^{-/-}$ mice (Guillemot et al., 1993), it seemed likely that neural crest-derived cells in the esophagus may have different trophic requirements and respond to different signaling pathways from other enteric crest-derived cells. However, the current study showed that, like intestinal crest-derived cells, crest-derived cells in the esophagus showed neurite outgrowth and migratory responses to both neurturin and GDNF. Thus, at least in the assays performed in this study, esophageal crest-derived cells behaved like other enteric crest-derived cells, although the decline in the GDNF-induced migratory response occurred in the esophagus before more caudal regions of the gut.

Most esophageal neurons are dependent on Ret

The reported presence of neurons only in the esophagus of *Ret*$^{-/-}$ mice, and the absence of neurons only from the esophagus of *Mash1*$^{-/-}$ mice (Guillemot et al., 1993), has lead to the idea that neural crest cells that migrate into the esophagus have different trophic requirements from neural crest cells that colonize the rest of the gut (Durbec et al., 1996). However, Ret appears to be downstream of Mash1 in signaling cascades (Lo et al., 1998), and if the development of esophageal neurons were Mash1-dependent but Ret-independent, it would suggest that there is a signaling pathway downstream of Mash1, other than Ret, that is essential for esophageal neuron development. However, our study showed that there are very few neurons in the esophagus of E18.5 *Ret*$^{-/-}$ mice and very few neural precursors (Phox2b^{+} cells) in the esophagus of E11.5 *Ret*$^{-/-}$ mice. It therefore seems likely that few neural crest cells ever migrate into the esophagus of embryonic *Ret*$^{-/-}$ mice, and hence Ret signaling is required by essentially all neural crest-derived cells destined to colonize the gut. The small number of neural crest cells that reach the esophagus and stomach probably only do so because they do not have to migrate very far to reach these gut regions. Interestingly, although gut neural crest stem cells are present in the esophagus of *Ret*$^{-/-}$ mice, there are four times more stem

cells in the esophagus of wild-type mice (Iwashita et al., 2003).

There are different types of myenteric neurons in the esophagus. In the current study, we showed that 40% of Hu^+ cells expressed NOS in E18.5 heterozygote and wild-type mice. However, of a total of 121 Hu^+ neurons observed in the esophagus of three $Ret^{-/-}$ mice, only 1 was NOS^+. This suggests either that the differentiation of all esophageal neurons is delayed in the absence of Ret signaling, or that Ret is specifically required for the development of NOS neurons. In the developing esophagus, other major classes of myenteric neurons (e.g., cholinergic neurons) do not develop until after birth (Sang et al., 1999), so we were unable to examine the appearance of other sub-populations of neurons in Ret null mice.

The main difference between esophageal neurons and other gastrointestinal neurons appears to be their dependency on Mash1; the development of all esophageal neurons is Mash1-dependent, whereas only some sub-populations of neurons in the intestine require Mash1 for their development (Blaugrund et al., 1996). In addition to Mash1, *Ret* can also be activated by Phox2b (Lo et al., 1998; Pattyn et al., 1999). $Phox2b^{-/-}$ mice lack enteric neurons in all regions of the gastrointestinal tract, including the esophagus (Pattyn et al., 1999). It therefore seems likely that both Mash1 and Phox2b are required for *Ret* activation in esophageal neuron precursors, but only Phox2b is required for *Ret* activation in most intestinal neuron precursors.

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